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A Proline-Rich Sequence Unique to MEK1 and MEK2 Is Required for Raf Binding and Regulates MEK Function

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Mammalian MEK1 and MEK2 contain a proline-rich (PR) sequence that is absent both from the yeast homologs Ste7 and Byr1 and from a recently cloned activator of the JNK/stress-activated protein kinases, SEK1/MKK4. Since this PR sequence occurs in MEKs that are regulated by Raf family enzymes but is missing from MEKs and SEKs activated independently of Raf, we sought to investigate the role of this sequence in MEK1 and MEK2 regulation and function. Deletion of the PR sequence from MEK1 blocked the ability of MEK1 to associate with members of the Raf family and markedly attenuated activation of the protein in vivo following growth factor stimulation. In addition, this sequence was necessary for efficient activation of MEK1 in vitro by B-Raf but dispensable for activation by a novel MEK1 activator which we have previously detected in fractionated fibroblast extracts. Furthermore, we found that a phosphorylation site within the PR sequence of MEK1 was required for sustained MEK1 activity in response to serum stimulation of quiescent fibroblasts. Consistent with this observation, we observed that MEK2, which lacks a phosphorylation site at the corresponding position, was activated only transiently following serum stimulation. Finally, we found that deletion of the PR sequence from a constitutively activated MEK1 mutant rendered the protein nontransforming in Rat1 fibroblasts. These observations indicate a critical role for the PR sequence in directing specific protein-protein interactions important for the activation, inactivation, and downstream functioning of the MEKs.

The mitogen-activated protein (MAP) kinases or extracellular signal-regulated kinases (ERKs), a family of conserved serine/threonine kinases, are implicated in the control of cell growth and division and cell differentiation (8, 12, 13, 66). MAP kinases are dependent on tyrosine and threonine phosphorylation for activity (3, 56), reactions that are catalyzed by a family of dual-specificity kinases called MAP kinase kinases or MEKs (MAP kinase or ERK kinases). MEKs are regulated by reversible serine/threonine phosphorylation (24), and a number of kinases that can function as MEK activators in vitro have been described. These include MEK kinases, isolated by virtue of their homology to the yeast proteins Ste11 and Byr2 (41), c-Mos (57), and a number of unidentified MEK activators detected in fractionated extracts from agonist-stimulated cells (27, 55, 58). In particular, there is much biochemical (1, 7, 15, 29, 33, 37, 50, 58, 69), genetic (2, 19, 26, 29, 32, 36, 37, 47, 64, 68), and regulatory (33, 40, 49, 58, 62, 69) evidence to suggest that members of the Raf family play an important role in the activation of MEK in vivo. Specifically, partially purified preparations of c-Raf-1 and B-Raf can activate MEK1 in vitro (1, 7, 15, 22, 29, 37, 43, 58); a Raf homolog has been shown to function downstream of receptor tyrosine kinases but upstream of MAP kinase in *Caenorhabditis elegans* (26), and overexpression of mutant, inactive forms of c-Raf-1 has been shown to prevent MAP kinase activation, presumably by a dominant-negative mechanism (64).

A related kinase cascade is induced in response to cell stress (23, 25, 38, 60). This results in activation of stress-activated protein (SAP) kinases (38) or Jun kinases (JNKs [17]), which are distantly related to the MAP kinases. JNK/SAP kinases are also dependent upon tyrosine and threonine phosphorylation for activity (17, 39), and recent data indicate that SEK1/MKK4, a kinase structurally related to MEK, can catalyze these reactions (18, 63, 75). Overexpression of activated MEK kinase, but not oncogenic Raf, results in efficient activation of the JNK/SAP kinases (48, 75), demonstrating that the cell can specifically regulate signalling through the MAP kinase or JNK/SAP kinase pathways.

At least four MEKs/SEKs have been cloned from mammalian sources (11, 18, 63, 73, 74, 78), and these show considerable similarity to each other and the yeast homologs Ste7 and Byr1 (11, 73, 74). However, the mammalian MEKs differ from the yeast homologs in that they contain a proline-rich (PR) sequence between conserved kinase subdomains IX and X (11, 73, 74); this sequence is also absent from SEK1/MKK4 (18, 63). Significantly, Ste7, Byr1, and SEK1 are activated by structurally related kinases, Ste11, Byr2, and MEK kinase, respectively (20, 41, 51), and furthermore, Byr1 is not activated by Raf in vivo (32). These data suggest a role for the PR sequence in recognition and activation by Raf family kinases and raise the possibility that this sequence helps control cross talk between structurally related but functionally distinct kinase cascades.

MEK1 and MEK2 exhibit considerable similarity within the PR sequence described above. However, of interest are two potential phosphorylation sites unique to MEK1, at threonine residues 286 and 292 (73, 74). Phosphorylation of MEK1 on these residues has been reported in mitotic HeLa cells and can be recapitulated in vitro with purified cyclin B-p34^{cdc2} (59). This modification is reported to inactivate MEK1 catalytic function in vitro (59). Inactivation may result from phosphorylation of threonine 286, since phosphorylation of threonine 292 in vitro with MAP kinase has no discernible effect on activation of MEK1 by Raf- or MEK1-catalyzed phosphorylation of MAP kinase (45, 61). However, a previous report from

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this laboratory indicates that threonine 292 plays an important role in directing protein-protein interactions (34). Specifically, MEK1 can associate with immobilized GTP-Ras and Raf in vitro (30, 34, 50), whereas neither MEK1 T292A (in which threonine 292 is mutated to alanine) nor MEK2, which lacks a phosphorylatable residue at the corresponding position, is able to form the ternary complex (34).

Mutationally activated MEK1 is oncogenic in fibroblasts (10, 44) and causes differentiation in PC12 cells (10). Oncogenic transformation of NIH 3T3 cells by mutationally activated MEK1 may be independent of Ras (10), since microinjection of an inhibitory anti-Ras antibody does not block DNA synthesis in these cells (10). The differentiating activity of mutationally activated MEK1 requires functional MAP kinase (10), but it is not clear whether activation of MAP kinase is alone sufficient for transformation and/or differentiation.

In this paper, we present data supporting a role for the PR sequence in directing complex formation between Raf and MEK1. We also show that disruption of this complex correlates with poor activation of MEK1 in vitro and in vivo, providing evidence that this specific protein-protein interaction regulates stimulation of MEK1 by the Raf family of MEK activators. However, deletion of the PR sequence does not preclude activation by a recently described (58) but unidentified MEK activator(s) present in fractionated fibroblast extracts, indicating that this sequence is dispensable for interaction of MEK1 with other classes of upstream activators. We also present evidence that threonine 292, a MEK1-specific phosphorylation site, modulates the kinetics of inactivation of MEK1 following stimulation by growth factors. Finally, we show that deletion of the PR sequence from mutationally activated forms of MEK1 inhibits the transforming activity of these proteins, indicating that this sequence plays an important role in downstream signalling by MEK1.

MATERIALS AND METHODS

Cell culture and transfection. CCL39 and Rat1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 5% (vol/vol) each bovine serum and fetal bovine serum. Serum deprivation was for 16 to 20 h in DMEM containing 0.1% (vol/vol) fetal bovine serum. Transfections (2 μg of plasmid DNA) were performed with appropriate mock controls, using Lipofectin (GIBCO-BRL, Grand Island, N.Y.) according to the manufacturer's instructions. After 48 h, cells were split into medium containing G418 to generate stably transfected populations. We routinely obtained ~400 to 500 G418-resistant colonies per μg of plasmid DNA. Colonies were picked and expanded or were pooled to yield transfected populations. Transient transfections were performed in the same fashion except that cells were harvested after 48 h. For some experiments, cells were serum deprived for the last 16 h of this incubation.

Plasmid construction and mutagenesis. Rat MEK1 (74) and MEK2 (73) cDNAs (a gift of J. Wu and T. W. Sturgill) were tagged at the N terminus with the influenza virus hemagglutinin (HA) epitope. Briefly, adenosine deaminase was excised from pLNCAL7 (53) and replaced with an oligonucleotide specifying a translation initiation codon, HA tag, and unique *Not*I site, to yield vector pCMV-HA. MEK inserts were generated by PCR amplification of cDNAs and ligated to *Not*I-digested pCMV-HA, fusing the amino acid sequence MDTKY PYDVHDYAAA in frame with codon 2 of MEK1 or MEK2. Mutagenesis was performed in Bluescript (Stratagene, La Jolla, Calif.), using a Transformer site-directed mutagenesis kit (Clontech, Palo Alto, Calif.). Deletion of MEK1 residues 270 to 307 was achieved by removal of an internal *Bst*XI-*Nco*I fragment. This replaces lysine 269 with an asparagine residue and deletes residues 270 to 307. Deletion of MEK1 residues 32 to 51 was achieved by removal of an internal *Stu*I fragment (44). Mutants were confirmed by sequencing following subcloning to pCMV-HA. Oligonucleotide sequences are available on request. pZIPneo H-ras L61 (16) was a kind gift of C. Der. For expression of MEK proteins in *Escherichia coli*, appropriate fragments were subcloned to pGEX 2T (Pharmacia, Uppsala, Sweden).

Cell stimulation and extraction for kinase assays. Serum-deprived cells were stimulated with 100 ng of epidermal growth factor (EGF; Upstate Biotechnology, Inc., Lake Placid, N.Y.) per ml, 1 U of thrombin (Sigma, St. Louis, Mo.) per ml, or 10% (vol/vol) fetal bovine serum for the indicated times at 37°C. Plates were washed with ice-cold calcium- and magnesium-free phosphate-buffered saline (PBS) before extraction in lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.5; 4°C], 150 mM NaCl, 50 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM EDTA, 1% [vol/vol] Nonidet P-40, 3 mM benzamidine, 5 μg of leupeptin per ml, 20 μg of aprotinin per ml, 40 mM *p*-nitrophenylphosphate, 1 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride [PMSF]). Extracts were clarified at 14,000 × *g* for 15 min at 4°C. Protein concentration was estimated by Coomassie dye binding, using reagent from Bio-Rad (Hercules, Calif.).

Immunoprecipitations and immune-complex kinase assays. Monoclonal anti-HA antiserum 12CA5 (Babco, Berkeley, Calif.) was preadsorbed to protein A-agarose (Boehringer Mannheim, Indianapolis, Ind.) before incubation with 200 μg (Rat1 cells) or 500 μg (CCL39 cells) of extract protein for 2 h at 4°C. Immunoprecipitates were washed three times with lysis buffer and twice with 25 mM HEPES-NaOH–10 mM $Mg(CH_3COO)_2$ (pH 7.5) at 4°C. MEK assays were performed at 30°C for 10 to 15 min in a final volume of 40 μl containing 25 mM HEPES-OH (pH 7.5; 4°C), 10 mM $Mg(CH_3COO)_2$, 1 mM dithiothreitol, 0.1 mM [γ-^{32}P]ATP (2,000 to 8,000 cpm/pmol), and 2 μg of purified kinase-defective MAP kinase (K52R). All assays were within the linear range. Reactions were terminated with sample buffer, and products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to nitrocellulose. Incorporation into K52R was quantitated by Cerenkov counting.

Column chromatography and MEK activator assays. Fractionation of NIH 3T3 extracts and assay of resulting fractions for MEK and MEK activator activity were performed as described previously (7, 58). One unit of MEK activator is that activity which increases the K52R-phosphorylating activity of 200 ng of recombinant glutathione *S*-transferase (GST)–MEK1 by 1 pmol/min in the described assay.

Coimmunoprecipitation assays. Cells transiently or stably transfected with expression constructs were washed in ice-cold PBS and lysed in p21 buffer (50) supplemented with 1% (vol/vol) Nonidet P-40, 10 mM NaF, 2 mM $Na_4P_2O_7$, 3 mM benzamidine, 5 μg of leupeptin per ml, 5 μM pepstatin A, 20 μg of aprotinin per ml, and 1 mM PMSF. Cells were scraped, and extracts were clarified by centrifugation at 14,000 × *g* for 15 min at 4°C. Protein A-purified anti-HA antibody 12CA5 was preadsorbed to protein A-agarose before incubation with cell extracts for 2 to 3 h at 4°C. Immunoprecipitates were washed four times with lysis buffer, resuspended in SDS-PAGE sample buffer, and resolved by SDS-PAGE. Gels were transferred to nitrocellulose and probed with antisera specific for c-Raf-1 and HA tag.

Immunoblotting. Nitrocellulose membranes were processed as described previously (7). Anti-HA monoclonal 12CA5, anti-c-Raf-1 (raised against 12 C-terminal residues; Santa Cruz Biotechnology Inc., Santa Cruz, Calif.), and anti-B-Raf (raised against 19 C-terminal residues; Santa Cruz Biotechnology Inc.) antisera were used at a concentration of 0.1 μg/ml. Primary antibody was detected by using either protein A-horseradish peroxidase conjugate and enhanced chemiluminescence reagents (Amersham, Arlington Heights, Ill.) according to manufacturer's instructions or ^{125}I-protein A (ICN, Costa Mesa, Calif.).

Purification of GST fusion proteins. Recombinant *E. coli* DH5α cells were grown in Luria-Bertani–ampicillin broth at 37°C until the A_{550} was ~1.0 (1-cm path length). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.03 mM, and cultures were induced at 25°C for 12 h. Cells were pelleted, frozen in liquid nitrogen, and stored at −70°C. Pellets were lysed by sonication in 50 mM Tris-HCl–150 mM NaCl–0.1% (vol/vol) Triton X-100 (pH 8.0; 4°C) supplemented with 25 mM EDTA, aprotinin, benzamidine, leupeptin, and PMSF, and fusion proteins were purified by affinity chromatography on glutathione-Sepharose (Pharmacia). Fusion proteins immobilized on glutathione-Sepharose were stored at 4°C. Alternatively, proteins were eluted by incubation with 50 mM Tris-HCl–20 mM glutathione (pH 8.0) for 15 min at 4°C, dialyzed overnight against 50 mM Tris-HCl–1 mM dithiothreitol (pH 8.0), and stored at −70°C. MEK fusion proteins were approximately 50 to 80% full length as judged by SDS-PAGE and Coomassie blue staining. The concentration of full-length protein was assessed by comparison with bovine serum albumin standards.

Pull-down experiments. Immobilized GST-MEK fusion proteins (~1 μg) or GST alone (~5 μg) was incubated with 1 to 4 mg of protein from untransfected CCL39 cells (prepared as described for the coprecipitation assays). After ~3 h at 4°C, complexes were washed four times with modified p21 buffer (see above), solubilized in SDS-PAGE sample buffer, and resolved by SDS-PAGE. Complexes were analyzed by Western blotting (immunoblotting).

Metabolic labelling and phosphopeptide mapping. Confluent cultures in 100-mm-diameter plates were labelled in 4.5 ml of labelling medium (phosphate-free DMEM [GIBCO-BRL] supplemented with pyruvate and 10% [vol/vol] conditioned regular medium) containing 1 mCi of carrier-free $^{32}P_i$ per ml for ~16 h. Extract preparation and anti-HA immunoprecipitation were performed as described above except that all of the extract from each plate was incubated with anti-HA antisera. Immunoprecipitates were washed five times with lysis buffer and four times with 0.1 M Tris-HCl–0.5 M LiCl (pH 7.5). Precipitates were resuspended in SDS-PAGE sample buffer and resolved by SDS-PAGE. After transfer to nitrocellulose, tryptic phosphopeptide mapping and phosphoamino acid analysis were performed essentially as previously described (7).

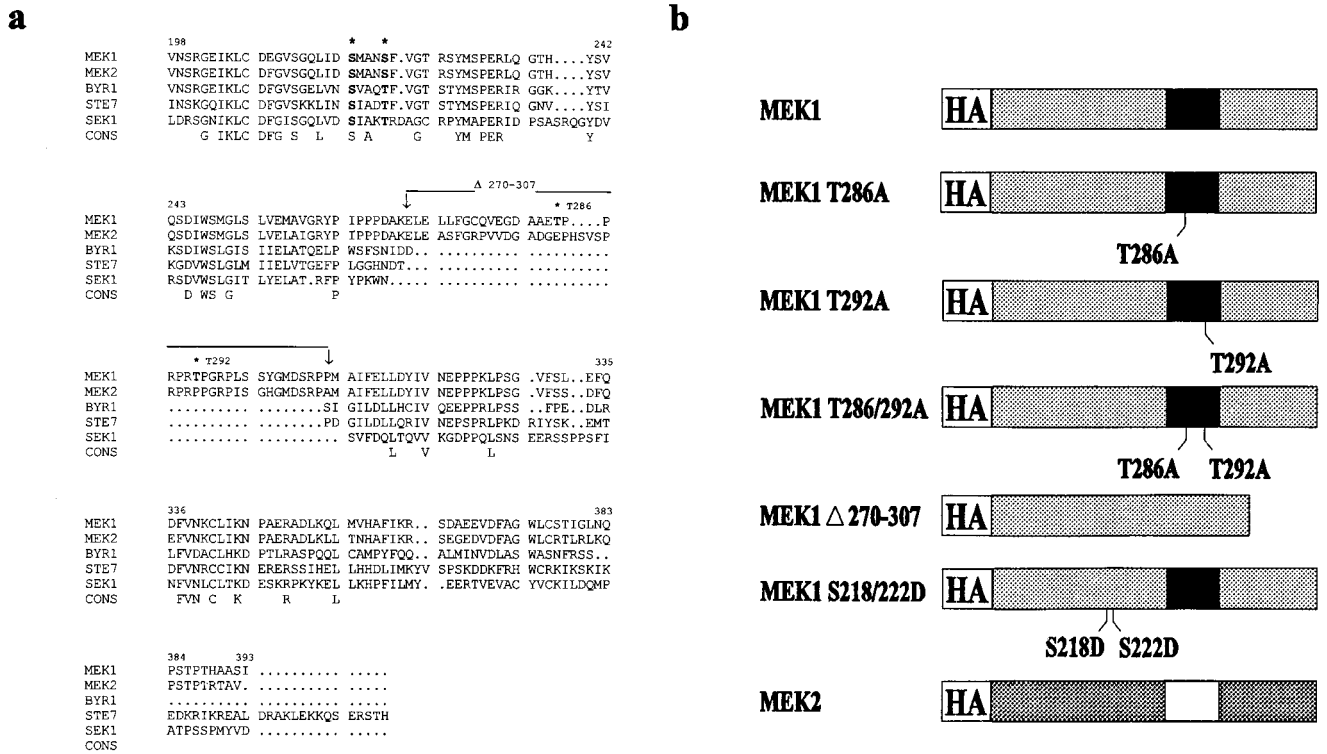

FIG. 1. A PR sequence unique to MEK1 and MEK2. (a) Sequence comparison of MEK1 (rat), MEK2 (rat), Ste7 (*Saccharomyces cerevisiae*), Byr1 (*Schizosaccharomyces pombe*), and SEK1 (mouse). Sequences are in the single-letter code and were compiled by using the PILE-UP program (Wisconsin Genetics Computer Group). Sequence alignment was optimized by eye, and gaps introduced into the sequences during compilation are illustrated by dots. Sites of phosphorylation mentioned in the text are denoted with asterisks; threonine 286 and threonine 292, phosphorylation sites specific to the MEK1 PR sequence, are labelled T286 and T292, respectively. A bar above the sequence indicates the extent of the deletion in the MEK1 Δ270-307 mutant (the ΔPR sequence mutant). Cons, consensus. (b) Schematic of MEK mutations used in this study. HA denotes the N-terminal HA epitope tag, and the PR sequence is illustrated by a black box (MEK1) or white box (MEK2). Point mutations are indicated below each schematic.

RESULTS

A PR sequence in MEK1 is required for association with c-Raf-1. Recent data demonstrate that GTP-Ras forms a complex in vitro with c-Raf-1 and MEK1 (34, 50) and that Raf is able to physically link Ras and MEK1 (70). Since MEK1 T292A (in which threonine 292 is mutated to alanine) and MEK2 (which has a proline at the residue corresponding to T292 in MEK1) both fail to bind to immobilized Ras-GTP (34), we hypothesized that the PR sequence, of which threonine 292 is part, regulates the association of MEK1 with c-Raf-1. To test this hypothesis, we constructed epitope-tagged MEK variants for coimmunoprecipitation assays (Fig. 1b). MEK1 T286A and MEK1 T292A contain point mutations at known phosphorylation sites, threonines 286 and 292 respectively, within a PR sequence of MEK1 (59, 61) (see below). These mutants, and the double mutant MEK1 T286/292A, therefore resemble MEK2, which lacks phosphorylatable residues in the corresponding positions (Fig. 1a). A fourth mutant, MEK1 Δ270-307, deleted of residues 270 to 307 of the PR sequence, was also constructed (Fig. 1). This deletion mutant more closely resembles the yeast MEK homologs Ste7 and Byr1 and a recently cloned activator for the JNK/SAP kinases, SEK1/MKK4 (18, 63), all of which lack a similar PR sequence (Fig. 1a).

Epitope-tagged MEK constructs were transiently transfected into CCL39 fibroblasts as described in Materials and Methods. After 48 h, cells were lysed and subjected to immunoprecipitation with antisera specific for the HA epitope tag. After resolution by SDS-PAGE, immunoprecipitates were probed with anti-HA and anti-c-Raf-1 antisera (Fig. 2a). Similar levels of HA-tagged MEKs were detectable in all transfections with the exception of the negative controls (mock and empty vector). Likewise, c-Raf-1 protein was found in anti-HA immunoprecipitates from HA-tagged MEK1-transfected cells (lane 2) but not empty vector (lane 1) or mock immunoprecipitations (lane 8), consistent with an association between MEK1 and c-Raf-1 (30). Significantly, we were unable to detect c-Raf-1 coprecipitating with MEK1 Δ270-307 (lane 6), suggesting that these residues are required for stable association with c-Raf-1. However, point mutations at known phosphorylation sites, threonines 286 and 292, within the MEK1 PR sequence did not detectably affect coprecipitation of c-Raf-1 (lanes 3 to 5); furthermore, c-Raf-1 was also found in immunoprecipitates derived from cells transfected with HA-tagged MEK2 (lane 7). Deletion of the corresponding PR sequence in MEK2 also abolishes coimmunoprecipitation of c-Raf-1 (data not shown). These data indicate that c-Raf-1 can form complexes with both MEK1 and MEK2 and that integrity of the MEK PR sequence is necessary for coimmunoprecipitation. However, phosphorylation of MEK1-specific sites within the PR sequence is not required for complex formation under these conditions. Taken together with our previous data (34), this finding suggests that association of MEK with Raf is not itself sufficient for association of MEK with GTP-Ras, since neither MEK2 nor MEK1 T292A was found in association with immobilized GTP-Ras under similar conditions (34). Comparable results were obtained for CCL39 cells stably transfected with the epitope-tagged MEK constructs (data not shown):

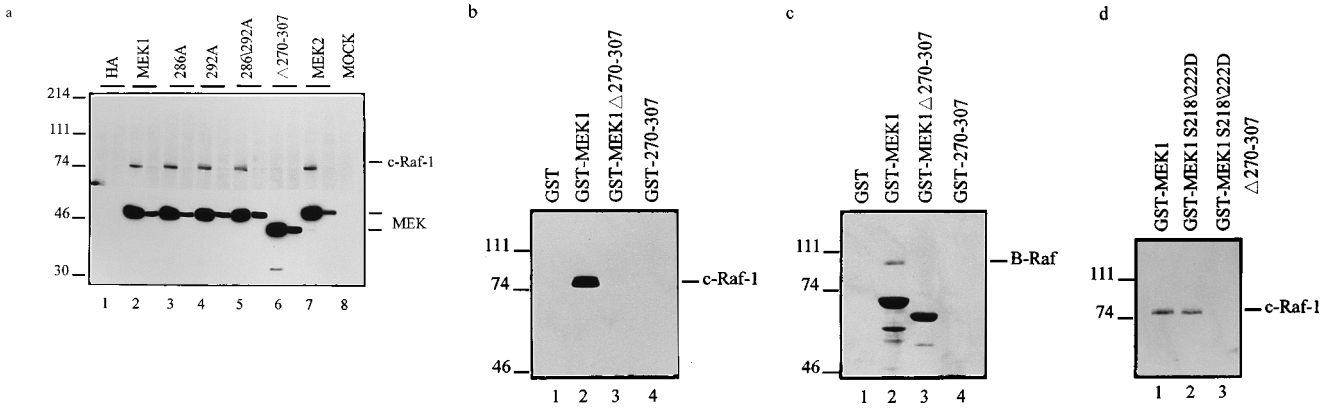

FIG. 2. The proline-rich sequence of MEK1 is necessary for association with c-Raf-1 and B-Raf. (a) HA-tagged MEK constructs were transiently transfected into CCL39 cells, and antiepitope immunoprecipitates were prepared after 48 h. Immunoprecipitates were resolved by SDS-PAGE and subject to immunoblot analysis with antiserum specific for c-Raf-1 (top of filter) or the HA tag (bottom of filter). Constructs transfected are indicated at the top, positions of molecular mass standards are shown in kilodaltons at the left, and the positions of c-Raf-1 and HA-tagged MEK are shown at the right. HA denotes empty vector transfection. Unlabelled lanes represent transfections carried out at higher plasmid concentrations, resulting in lower MEK expression. Δ270-307 is the PR sequence deletion mutant. (b to d) Immobilized GST or GST-MEK1 fusion proteins were incubated with CCL39 extract, and bound proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Filters were probed with antisera specific for c-Raf-1 (b and d) or B-Raf (c). Fusion proteins used, indicated at the top, are based on the wild-type enzyme (b and c) or a mutationally activated enzyme (d). Positions of molecular mass markers are shown in kilodaltons at the left, and the positions of c-Raf-1 and B-Raf are indicated at the right. The anti-B-Raf cross-reactive material migrating ahead of B-Raf in panel b is GST-MEK fusion protein. GST-270-307 is a fusion of MEK1 residues 270 to 307 (the PR sequence) with GST.

approximately 5 to 10% of the c-Raf-1 protein could be coprecipitated with epitope-tagged MEK. Quantitatively, coprecipitation of c-Raf-1 with MEK1 was not influenced by serum stimulation of quiescent cultures, although serum stimulation did result in coprecipitation of c-Raf-1 protein with reduced electrophoretic mobility (data not shown). This mobility shift is characteristic of c-Raf-1 from stimulated cultures and probably results from feedback phosphorylation events (62).

The importance of the MEK1 PR sequence in binding of c-Raf-1 was further investigated in pull-down experiments. Extracts prepared from untransfected CCL39 fibroblasts were incubated with immobilized GST-MEK fusion proteins as described in Materials and Methods, and bound proteins were analyzed by SDS-PAGE and immunoblotting (Fig. 2b). Consistent with the coimmunoprecipitation data, c-Raf-1 was pulled down by GST-MEK1 (lane 2) but not by GST-MEK1 Δ270-307 (lane 3) or GST alone (lane 1). However, MEK1 residues 270 to 307 appear insufficient for stable association with c-Raf-1, since a fusion between GST and these residues was unable to precipitate c-Raf-1 (lane 4). Thus, the PR sequence is necessary but not sufficient for stable association with c-Raf-1.

By comparison to the starting extract, approximately 5 to 10% of the c-Raf-1 was recovered with the immobilized GST-MEK1 (not shown); no anti-c-Raf-1 reactive material was seen if cell extract was omitted (data not shown). Qualitatively identical results were obtained after probing with antisera specific for B-Raf (Fig. 2c). Since B-Raf is a major MEK1 activator in CCL39 (data not shown) and other fibroblast cell lines (58), this interaction may be functionally significant.

To exclude the possibility that deletion of residues 270 to 307 causes denaturation of MEK1 and thereby precludes binding of c-Raf-1, we carried out similar pull-down experiments using a mutant form of MEK1 in which the sites of activating phosphorylation, serines 218 and 222 (1, 10, 31, 44, 54, 76, 79), are mutated to aspartate residues. MEK1 S218/222D (44, 76) and the corresponding deletion mutant, MEK1 S218/222D Δ270-307 (data not shown), exhibit constitutively elevated MEK activity when purified from *E. coli*, demonstrating that deletion of the PR sequence does not inactivate the catalytic function of MEK. GST-MEK1 S218/222D and GST-MEK1 S218/222D Δ270-307 were incubated with CCL39 extract and processed as described above. Catalytically active GST-MEK1 S218/222D Δ270-307 also failed to bind c-Raf-1 (Fig. 2d, lane 3), suggesting that deletion of residues 270 to 307 resulted in a defect in the binding properties of MEK1 without loss of catalytic activity. Note that MEK1 S218/222D retained the ability to complex with c-Raf-1 (Fig. 2d, lane 2).

The PR sequence is important for activation of MEK1 in vivo. CCL39 fibroblasts were transfected with HA-tagged MEK constructs or the corresponding empty vector, and populations were selected on the basis of their resistance to G418 as described in Materials and Methods. Confluent cultures were serum starved and then left untreated or stimulated with either EGF or thrombin for 5 min. After lysis, epitope-tagged MEK proteins were recovered by immunoprecipitation and used in an in vitro kinase reaction with purified MAP kinase as the substrate (Fig. 3a, top panel). Epitope-tagged wild-type MEK1 activity was detectable in untreated cells (lane 4) and robustly stimulated by pretreatment with either thrombin or EGF (lanes 5 and 6). In contrast, MEK activity was essentially undetectable in antiepitope immunoprecipitates prepared from serum-deprived CCL39 cells transfected with HA-tagged MEK1 Δ270-307 (lane 7) and was approximately 10-fold lower than wild-type level after stimulation with either thrombin or EGF (lanes 8 and 9) or fetal calf serum (data not shown). Blotting with antisera specific for the epitope tag confirmed that similar amounts of HA-tagged MEK were present in immunoprecipitates of the wild-type and mutant proteins (Fig. 3a, lower panel). These data indicate that deletion of residues 270 to 307 from MEK1 inhibits the in vivo activation of the enzyme, as measured by its ability to phosphorylate kinase-defective MAP kinase. However, note that because the basal activity of the MEK1 Δ270-307 protein was immeasurably low, it is possible that the fold stimulation of the mutant protein was comparable to the wild-type level. Importantly, robust activation of MEK1 Δ270-307 was seen following serum stimulation of transiently transfected CCL39 cells and in CCL39 cells transiently cotransfected with MEK1 Δ270-307 and oncogenic Ras (Fig. 3b). This result confirms that the deletion mutant can be

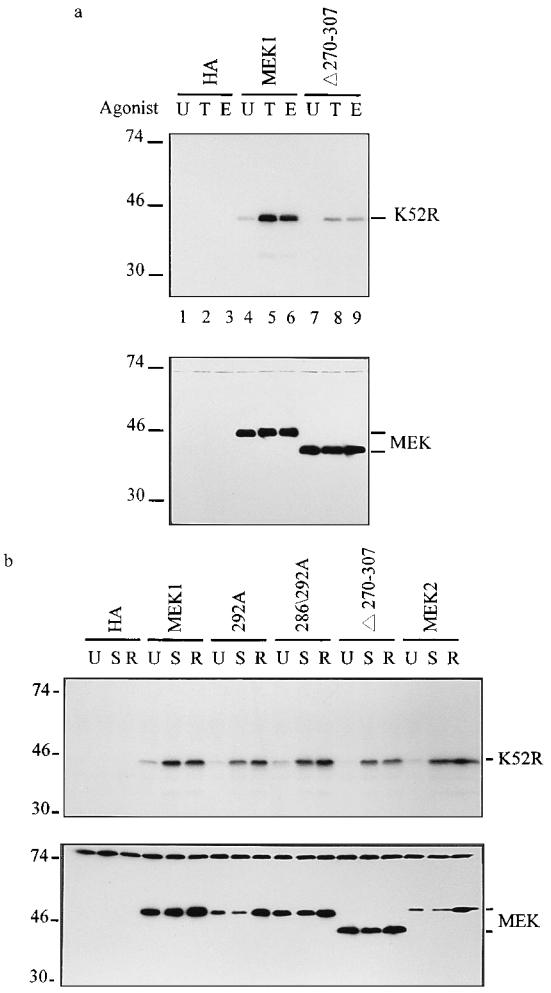

FIG. 3. The PR sequence is important for activation of MEK1 in vivo. (a) Populations of CCL39 cells stably transfected with the constructs indicated at the top were serum deprived and then left untreated (lanes U) or stimulated with thrombin (lanes T) or EGF (lanes E) for 5 min. HA-tagged MEK protein was recovered by immunoprecipitation and assayed with kinase-defective MAP kinase (K52R) and [γ-^{32}P]ATP as substrates (upper panel). Anti-HA immunoprecipitates shown in the top panel were subject to immunoblot analysis using anti-HA antiserum (lower panel). (b) CCL39 cells were transiently transfected with the HA-tagged MEK constructs (2 μg) indicated at the top, with (lanes R) or without (lanes U and S) 2 μg of the oncogenic H-Ras construct. After 30 h, all cultures were deprived of serum for ~16 h. Cultures were left untreated (lanes U and R) or stimulated with fetal bovine serum (lanes S) for 5 min before preparation of anti-HA immunoprecipitates. MEK assays (upper panel) were performed as described for panel a, and immunoprecipitates were subject to blotting with anti-HA antiserum (lower panel). K52R denotes the position of the phosphorylated substrate, and the positions of tagged MEK proteins are indicated at the right. Sizes of standards are indicated in kilodaltons at the left.

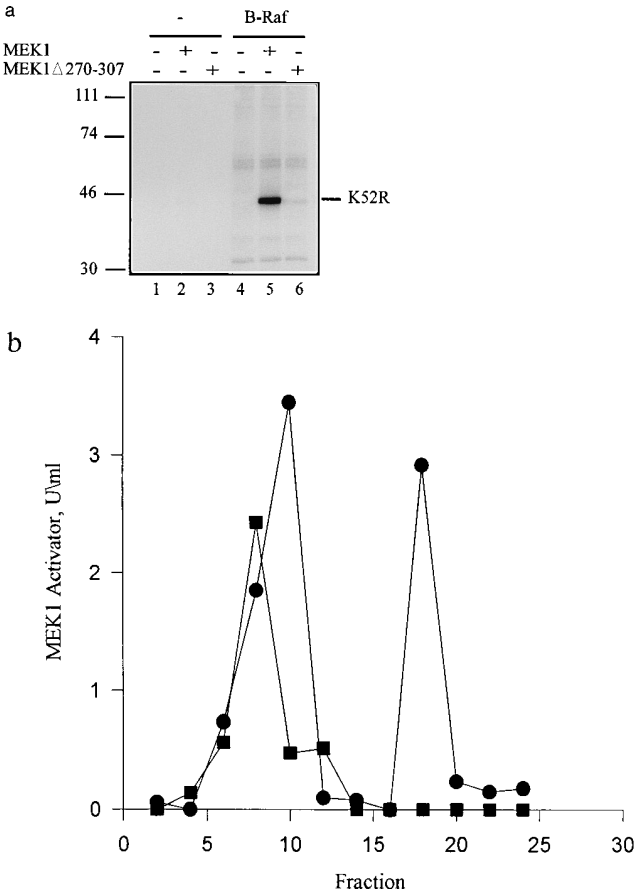

FIG. 4. The PR sequence is required for in vitro activation of MEK1 by some but not all MEK activators. (a) Recombinant GST-MEK1 or GST-MEK1 Δ270-307 (PR sequence deletion mutant) was incubated alone (−) or with B-Raf partially purified from bovine brain (B-Raf) in the presence of ATP and Mg^{2+} for 30 min. Kinase-defective MAP kinase (K52R) and [γ-^{32}P]ATP were added, and reactions continued for 10 min before termination with SDS-PAGE sample buffer. K52R denotes the phosphorylated substrate, and positions of molecular mass standards are indicated in kilodaltons at the left. (b) Quiescent NIH 3T3 fibroblasts were stimulated with fetal bovine serum for 2 min before fractionation on a Mono Q strong anion-exchange column (see Materials and Methods). Fractions were assayed for the ability to activate either GST-MEK1 (●) or GST-MEK1 Δ270-307 (■).

activated by factors that stimulate the wild-type enzyme; activation in the transient transfections presumably results from very high level expression within transfected cells, which would overcome the requirement for stable association with Raf.

The PR sequence is required for in vitro activation of MEK1 by some but not all MEK activators. Since our data suggest that deletion of residues 270 to 307 from MEK1 inhibits binding of c-Raf-1 and B-Raf and attenuates activation of the mutant protein in vivo in response to growth factors, we designed experiments to test whether proposed physiological activators of MEK1 could activate the MEK1 Δ270-307 deletion mutant in vitro. Wild-type GST-MEK1 or GST-MEK1 Δ270-307 was incubated with or without B-Raf (partially purified from bovine brain [7]) in the presence of ATP and Mg^{2+} prior to the addition of K52R and [γ-^{32}P]ATP (Fig. 4a). The recombinant MEK proteins had very low basal activity (lanes 2 and 3), and as expected, the activity of wild-type MEK1 could be markedly stimulated by B-Raf (lane 5). Interestingly, the activity of GST-MEK1 Δ270-307 was not stimulated by preincubation with B-Raf (lanes 6), indicating that sequences or structural elements within this deletion are necessary for recognition and activation by B-Raf.

Recently, using a biochemical approach to identify and quantitate MEK1 activators in fibroblasts (58), we reported the existence of three chromatographically separable MEK activators in soluble, detergent-free extracts. A major activity was identified as B-Raf, while two other activities remain unidentified (58). These activities are immunologically distinct from previously described MEK activators, and one appears to be insulin specific (58). Hence, we undertook experiments to compare the in vitro activation of GST-MEK1 and GST-MEK1 Δ270-307 by fractionated fibroblast extracts (Fig. 4b). Serum-

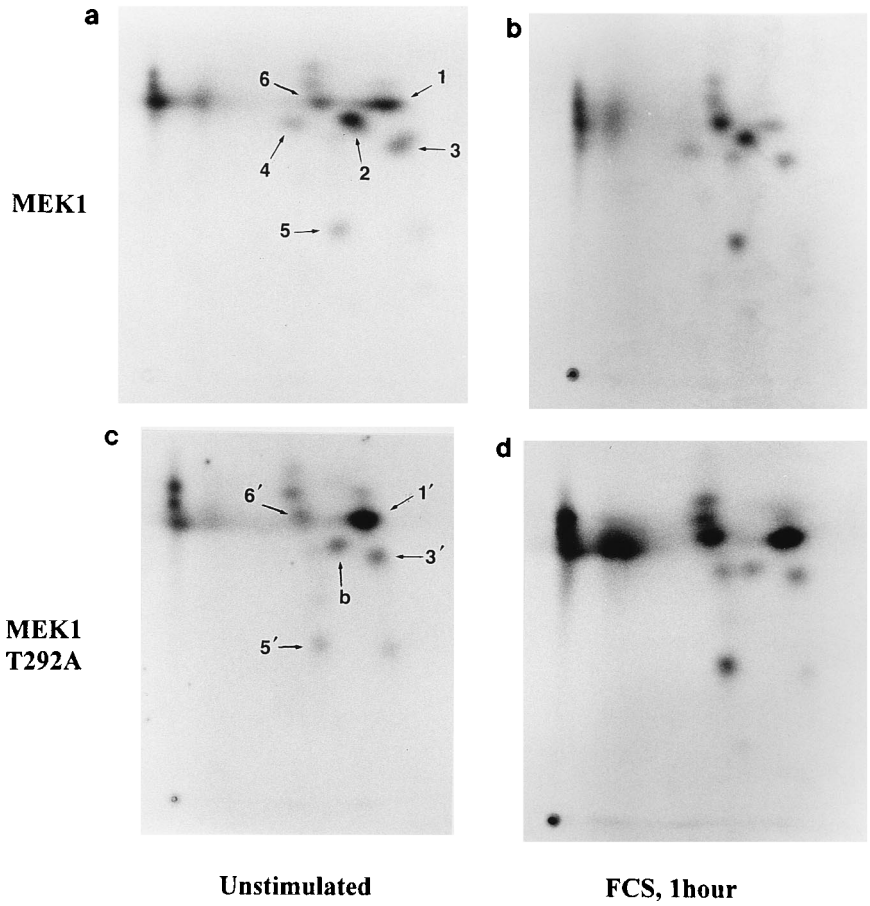

FIG. 5. MEK1 is constitutively phosphorylated on threonine 292. CCL39 cells stably expressing epitope-tagged MEK1 (a and b) or MEK1 T292A (c and d) were labelled metabolically with $^{32}P_i$ as described in Materials and Methods. Cultures were left untreated (a and c) or stimulated with fetal calf serum (FCS) (b and d) for 1 h prior to lysis. Tagged MEK proteins were purified by immunoprecipitation and digested with trypsin, and resulting phosphopeptides were resolved by two-dimensional thin-layer electrophoresis and ascending chromatography. The origin is in the lower left corner, with anode at the left and cathode at the right. Highlighted peptides (see text) were recovered from the plate and subjected to phosphoamino acid analysis and Edman degradation (see Table 1).

stimulated NIH 3T3 cells were fractionated by ion-exchange chromatography as previously described (58). Under these conditions, B-Raf elutes from the Mono Q column at ~200 mM NaCl, in fractions 15 to 20. The unknown MEK activator, which we will term peak I, elutes at ~50 mM NaCl, in fractions 5 to 12 (58); a similar activity has been observed in PC12 cell extracts (55). Column fractions were assayed for the ability to activate recombinant MEK1 and MEK1 Δ270-307, and as expected from the preceding data, MEK1 Δ270-307 was not activated by B-Raf. Importantly, both wild-type MEK1 and the deletion mutant were activated by the peak I activator, suggesting that this activator recognizes MEK1 independently of the PR sequence.

Taken together, our data imply that stable association is a prerequisite for activation of MEK1 by B-Raf in vitro and that deletion of the PR sequence compromises activation of MEK1 in vivo. Some activation of the deletion mutant occurs in cells stimulated with purified agonists or serum (Fig. 3), and this may result from the activity of the unidentified MEK activator that becomes enriched in peak I following ion-exchange chromatography.

Phosphorylation of threonine 292 correlates with prolonged MEK1 activity. The PR sequence of MEK1 contains two potential phosphorylation sites, threonine 286 and threonine 292, not present in MEK2 (Fig. 1a). A potential MAP kinase site is present in the C termini of both MEK1 (threonine 386) and MEK2 (threonine 404; Fig. 1a). Threonines 292 and 386 of MEK1 may be utilized in vivo (59, 61); furthermore, they can be modified in vitro by specific protein kinases (45, 59, 61). A role for threonine 292 (and threonine 386) phosphorylation in feedback inhibition of MEK1 has been proposed by Brunet et al. (5), but evidence for direct inhibition of MEK1 activity is lacking (45, 61). We undertook experiments to address the role of threonine 292 phosphorylation in regulation of MEK1 versus MEK2 activity in response to cell stimulation. HA-tagged MEK1 was labelled metabolically with $^{32}P_i$, purified by immunoprecipitation, and digested with trypsin as described in Materials and Methods. Labelling was performed under conditions of low serum, and prior to lysis, some dishes were stimulated with fetal calf serum (see the legend to Fig. 5). Two-dimensional peptide maps of HA-tagged MEK1 are shown in Fig. 5. Individual phosphopeptides were recovered from the plates and subjected to phosphoamino acid analysis and Edman degradation. The results of this analysis, and candidate peptides, are presented in Table 1. The data for phosphopeptides 2 to 4 are consistent with phosphorylation of threonine 292 for the following reasons. First, trypsin cleavage of rat MEK1 is predicted to release a peptide (residues 292 to 324) in which threonine 292 is the N-terminal residue; Edman degradation of such a phosphopeptide, if threonine phosphor-

TABLE 1. Constitutive MEK1 phosphorylation at threonine 292

Spot	Hydrolysis[a]	Cycle no.[b]	Candidate tryptic peptide
1	S	7	^{206}LCDFGV<u>S</u>GQLIDSMANSFVGTR ^{235}LQGTHY<u>S</u>VQSDIWSMGLSLVEMAVGR ^{292}TPGRPL<u>S</u>SYGMDSRPPMAIFELLDYIVNEPPPK ^{325}LPSGVF<u>S</u>LEFQDFVNK
2	T = S	1, 7	^{292}<u>T</u>PGRPL<u>S</u>SYGMDSRPPMAIFELLDYIVNEPPPK
3	T >> S	1	^{292}<u>T</u>PGRPLSSYGMDSRPPMAIFELLDYIVNEPPPK
4	S > T	1, 7	^{292}<u>T</u>PGRPL<u>S</u>SYGMD<u>S</u>RPPMAIFELLDYIVNEPPPK
1′	S	ND	
3′	S	ND	
b	S	ND	

[a] S and T, phosphoserine and phosphothreonine, respectively.
[b] Edman degradation releases radioactivity at these cycle numbers; corresponding residues are underlined. ND, not done.

ylated, would release radioactivity after one cycle. This was the case for peptides 2 to 4. Inspection of the rat MEK1 sequence reveals no other predicted tryptic peptides in which a threonine residue occurs at the N terminus. Second, phosphopeptides 2 and 4 had one or more sites of serine phosphorylation in addition to the site(s) of threonine phosphorylation; the tryptic peptide beginning at threonine 292 has a serine residue at position 7, consistent with the Edman degradation data shown in Table 1. One other threonine residue (threonine 386) in rat MEK1 occurs six residues displaced from a serine residue (serine 392); trypsin is not predicted to generate a peptide with threonine 386 as the N-terminal residue; furthermore, mutagenesis of threonine 386 to alanine did not affect in vivo phosphorylation of peptides 2 to 4 (data not shown). Finally, since phosphopeptides 1, 2, and 4 migrate on a diagonal, they might be phosphoisomers of the same peptide (4). This would predict that phosphopeptides 1, 2, and 4 are mono-, di-, and triphosphorylated forms, respectively, of the peptide encompassing residues 292 to 324. Thus, phosphopeptide 1 would be phosphorylated only on serine 298 (i.e., position 7), phosphopeptide 2 would be phosphorylated on both threonine 292 and serine 298 (i.e., positions 1 and 7), and phosphopeptide 4 would be phosphorylated on threonine 292, serine 298, and an additional (serine) residue (Table 1). Additional serine residues (299 and 304) are present at positions 8 and 13 of the predicted peptide, but we did not observe release of radioactivity at cycle 8 or 13; phosphorylation of serine 304 may have been undetectable because of the number of cycles required and the relatively low amount of phosphopeptide used. Thus, phosphoamino acid composition, Edman degradation, and phosphopeptide mobility are indicative of, but do not prove, phosphorylation of the tryptic peptide spanning MEK1 residues 292 to 324.

To confirm phosphorylation of threonine 292, we constructed a MEK1 mutant in which threonine 292 was mutated to alanine (Fig. 1b). If phosphopeptides 1, 2, and 4 are mono-, di- and triphosphorylated forms of the peptide encompassing residues 292 to 324, mutagenesis of threonine 292 would result in loss of phosphopeptide 4 and concomitant appearance of a serine (S-298 and S-304)-phosphorylated peptide at or near the position of peptide 2. Similarly, peptides that were phosphorylated on threonine 292 and serine 298 (i.e., peptide 2) in the wild-type enzyme would now be predicted to run at the position of peptide 1, since these peptides now contain only serine 298. Examination of the peptide maps generated from metabolically labelled MEK1 T292A (Fig. 5) and phosphoamino acid analysis of the resulting tryptic phosphopeptides (Table 1) indicated that this was indeed the case. Hence, phosphopeptide 4 was absent (confirmed by mixing experiments; data not shown); a phosphopeptide with mobility similar to that of peptide 2 was present (spot b) and, importantly, contained only phosphoserine; label associated with phosphopeptide 1′ was increased relative to that of other phosphopeptides and was incorporated exclusively in phosphoserine residues. Taken together, these data suggest that threonine 292, serine 298, and, by inference, serine 304 are phosphorylated in HA-tagged MEK1 isolated from serum-deprived CCL39 fibroblasts. Phosphopeptide 3, which in MEK1 was found to contain predominantly phosphothreonine with a trace of phosphoserine, was not removed from the peptide maps by mutagenesis of threonine 292 to alanine. However, acid hydrolysis of phosphopeptide 3′ from MEK1 T292A released only phosphoserine; we hypothesize that phosphopeptide 3 as isolated from the wild-type protein is a mixture of monophosphorylated peptides phosphorylated on either threonine 292 or an unidentified serine residue. If this is the case, it is unclear why these peptides fail to comigrate with phosphopeptide 1. Note that phosphopeptide maps generated by using MEK1 T286A, in which a potential cyclin-dependent kinase site (59) is mutated, were indistinguishable from those generated with the wild type (data not shown); this result suggests that MEK1 is not efficiently phosphorylated on threonine 286 in the first hour following serum stimulation of CCL39 fibroblasts.

Having established that threonine 292 was phosphorylated under conditions of serum deprivation, we sought to investigate the effect of serum stimulation on labelling of this site. Cells expressing HA-tagged MEK1 or MEK1 T292A were labelled with $^{32}P_i$ and either left untreated or stimulated with fetal calf serum for time periods ranging from 1 to 60 min. No obvious change in labelling of MEK1 phosphopeptides 1 to 4 was observed (Fig. 5 and data not shown), although there was a reproducible increase in labelling of phosphothreonine-containing peptides 5 and 6 following serum stimulation. Similarly, labelling of phosphopeptides 1′, 3′, and b in MEK1 T292A was not grossly affected by serum stimulation, although there were increases in labelling of phosphothreonine-containing peptides 5′ and 6′ (Fig. 5). Phosphopeptides 5 and 6 disappear when threonine 386 is mutated to alanine (data not shown), suggesting that threonine 386 is a site of serum-stimulated phosphorylation. Notably, both threonine 292 and 386 are found within consensus MAP kinase phosphorylation sites, but only threonine 386 appears sensitive to serum-stimulated phosphorylation, under conditions in which MAP kinase activity is elevated. The kinase(s) responsible for phosphorylation of threonine 292 is not known, but our data would argue against a role for MAP kinase since this site was utilized under conditions (i.e., low serum) in which MAP kinase activity is low; furthermore, labelling did not increase when MAP kinase ac-

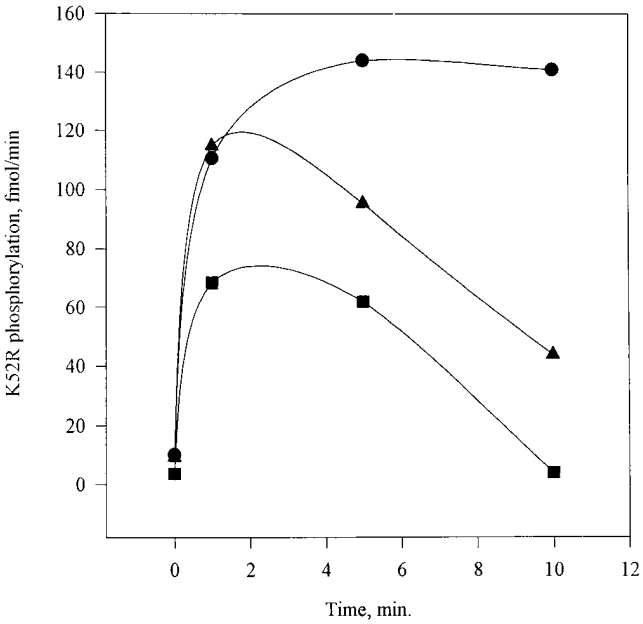

FIG. 6. Threonine 292 is required for sustained MEK1 activity in response to serum stimulation. CCL39 cells expressing HA-tagged MEK1 (●), HA-tagged MEK1 T292A (■), or HA-tagged MEK2 (▲) were serum deprived prior to stimulation with fetal bovine serum for the indicated times. Anti-HA immunoprecipitates were assayed as described in the legend to Fig. 3, and MEK activity was quantitated as described in Materials and Methods.

tivity was elevated. Threonine 292 phosphorylation appears not to result from overexpression of MEK1, since endogenous MEK1 protein purified from CCL39 yielded very similar phosphopeptide maps (data not shown).

Given the constitutive phosphorylation of threonine 292, a role for this residue in feedback inhibition of MEK1 activity (5) appears unlikely. However, it seemed possible that phosphorylation of this residue played a role in some other aspect of MEK1 activity or function. We therefore performed experiments to investigate the role of this site in regulation of MEK activity following serum stimulation. CCL39 fibroblasts stably transfected with epitope-tagged MEK constructs were serum deprived before stimulation with fetal calf serum for the indicated times. Tagged MEK protein was recovered by immunoprecipitation and assayed with recombinant kinase-defective MAP kinase as the substrate. As shown in Fig. 6, epitope-tagged MEK activity peaked in all cultures at 1 to 5 min following serum stimulation. However, MEK2 activity declined more rapidly than MEK1 activity after longer periods of stimulation. Notably, the mutant lacking the MEK1-specific phosphorylation site at position 292 was downregulated more rapidly than wild-type MEK1 and in this regard resembles MEK2. Blotting confirmed that similar amounts of MEK protein were present in all immunoprecipitates (data not shown), and these data were reproducible in at least five experiments. Since MEK2 lacks a phosphorylatable residue at the position corresponding to threonine 292, these data indicate an important role for threonine 292 phosphorylation in sustaining MEK1 activity following serum stimulation. However, it should be noted that these data were obtained in a system overexpressing the MEK proteins and that the precise role of this phosphorylation in regulation of the endogenous protein is not yet determined.

In summary, the PR sequences of MEK1 and MEK2 play a role in activation and inactivation of MEK activity in response to agonists. Activation of MEK1 appears to be linked to integrity of the PR sequence, whereas the persistence of MEK1 activity is coupled to the integrity of a phosphorylation site within this sequence. Threonine 292 of HA-tagged MEK1 is phosphorylated in serum-deprived cultures, and labelling of this site is not affected by serum stimulation. Hence, we believe it unlikely that threonine 292 is a site of MAP kinase mediated feedback inhibition, as has been proposed by others (5). Our data do not indicate a direct correlation between the rate of inactivation of MEK and the ability to bind Raf, since MEK1, MEK1 T292A, and MEK2 are all able to complex with c-Raf-1. However, there is a correlation between the longevity of MEK activity and the ability of MEK to complex with GTP-Ras (34). This observation will be discussed below.

The PR sequence is required for transformation by mutationally activated MEK1. The preceding data indicate an important role for the PR sequence in directing association with Raf family enzymes and in the regulation of MEK activity in response to growth factor stimulation in vivo. Since mutationally activated MEK1 is oncogenic for fibroblasts (10, 44) and causes differentiation of PC12 cells in vitro (10), we next investigated the role of the PR sequence in transformation by mutationally activated MEK1. Rat1 cells were transfected with appropriate expression vectors, and G418-resistant colonies were screened for morphological transformation (Fig. 7). Transfection with empty vector (Fig. 7a) or wild-type MEK1 (Fig. 7b) resulted in uniformly flat colonies of contact-inhibited cells. In contrast, transfection with epitope-tagged MEK1 S218/222D yielded morphologically transformed colonies (Fig. 7c and d) after G418 selection, in agreement with the findings of other laboratories (10, 44). The majority of the transformed colonies were similar to those resulting from transfection with oncogenic Ras (compare Fig. 7c and f), consisting of refractile, disorganized cells that grew in multilayers. Other colonies resulting from transfection with MEK1 S218/222D consisted of refractile, fusiform cells that were not contact inhibited (Fig. 7d). All colonies showing expression of MEK1 S218/222D were morphologically transformed (data not shown). Strikingly, transfection with a construct encoding the mutationally activated deletion mutant, MEK1 S218/222D Δ270-307, yielded only flat colonies of contact-inhibited cells (Fig. 7e). Similar results were obtained in three independent experiments; furthermore, no transformed colonies were observed when the number of G418-resistant colonies screened was increased from ~100 to ~500 (data not shown). Very similar results were obtained for the corresponding activated mutants of MEK2 (data not shown), and all of these data were reproduced in CCL39 cells (data not shown).

Previous experiments indicated that MEK1 S218/222D Δ270-307 had a five- to sevenfold-lower specific activity than MEK1 S218/222D when purified from cultured fibroblasts or recombinant *E. coli* (data not shown). It was therefore possible that a quantitative difference in kinase activity accounted for the different transforming potentials of these two mutant proteins. To test this possibility, we utilized observations made by Mansour et al. (44), who found that deletion of MEK1 residues 32 to 51 resulted in constitutively elevated activity and that this deletion could synergize with serine-to-glutamate mutations at the activating phosphorylation sites. Thus, the doubly activated MEK1 was ~4-fold more active than MEK1 in which only the activating phosphorylation sites had been mutated, and moreover was more transforming in cultured fibroblasts (44). Hence, we deleted residues 32 to 51 from MEK1 S218/222D Δ270-307 to yield MEK1 S218/222D Δ32-51 Δ270-307 and performed transformation assays in Rat1 cells as described above. In three independent experiments, we found no evi-

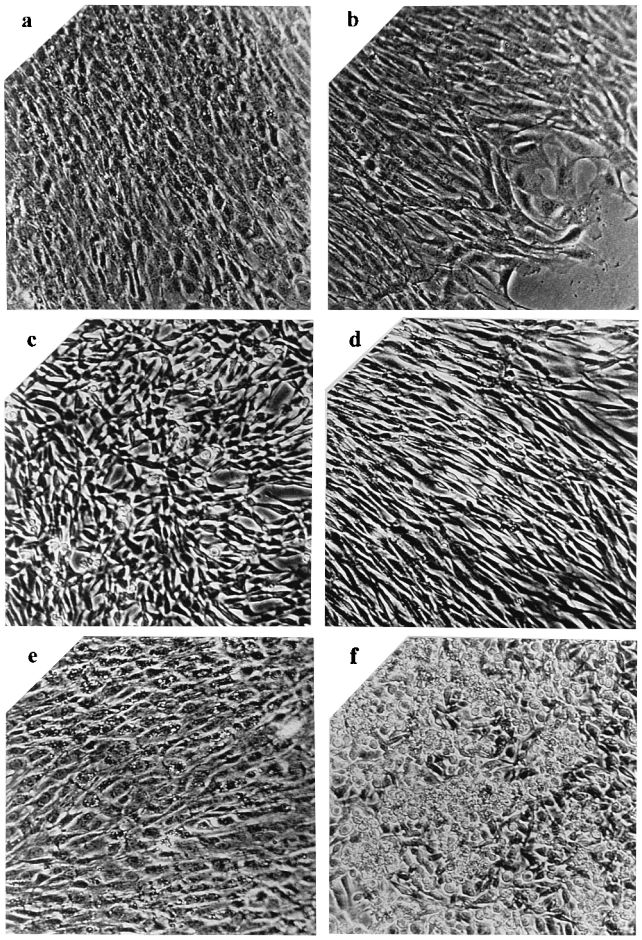

FIG. 7. The PR sequence is essential for morphological transformation by constitutively active MEK1. Rat1 cells were transfected with appropriate expression constructs, and colonies were photographed after selection with G418. Cells were transfected with empty vector (a), HA-tagged MEK1 (b), HA-tagged MEK1 S218/222D (mutationally activated) (c and d), HA-tagged MEK1 S218/222D Δ270-307 (mutationally activated, PR sequence deleted) (e), and oncogenic Ras (f).

dence of morphological transformation by MEK1 S218/222D Δ32-51 Δ270-307 under conditions in which MEK1 S218/222D was transforming (data not shown).

To extend these observations, we screened a number of colonies from each transfection for expression and activity of HA-tagged MEK proteins (Fig. 8). For MEK1 S218/222D, we chose to study a clone exhibiting florid transformation together with a fusiform-transformed clone. These were compared with two nontransformed clones expressing MEK1 S218/222D Δ270-307 and three nontransformed clones expressing MEK1 S218/222D Δ32-51 Δ270-307. Clones expressing empty vector, wild-type HA-tagged MEK1, and oncogenic Ras were included as controls. Cultures were serum deprived and either left untreated or stimulated with fetal bovine serum for 5 min. Anti-epitope immunoprecipitates were prepared from equivalent amounts of cell protein and assayed for MEK activity (Fig. 8, top panel) or blotted with antibody specific to the HA tag (lower panel) as described in Materials and Methods. MEK activity was quantitated by counting ^{32}P incorporated into the MAP kinase substrate. HA-tagged MEK activity recovered in precipitates from Rat1 cells fully transformed with MEK1 S218/222D was constitutive and 40- to 60-fold higher than the activity recovered from a clone expressing similar amounts of wild-type HA-tagged MEK1. The fusiform-transformed clone expressed less MEK1 S218/222D protein and exhibited somewhat lower MEK activity (~12-fold greater than wild-type activity) but was morphologically transformed nonetheless. Deletion of the PR sequence from MEK1 S218/222D rendered the protein nontransforming but, as mentioned above, also reduced MEK specific activity (Fig. 8, upper and lower panels). However, the three morphologically normal clones expressing MEK1 S218/222D Δ32-51 Δ270-307 contained epitope-tagged MEK activity comparable to that recovered from the fully transformed MEK1 S218/222D clone, demonstrating that constitutively elevated MEK activity is insufficient to elicit morphological transformation in the absence of the PR sequence. Qualitatively identical results were obtained upon screening colonies resulting from transfection with MEK2 S222/226D and the corresponding deletion mutant MEK2 S222/226D Δ273-315; furthermore, all of these data were reproduced in CCL39 cells (data not shown). Control experiments demonstrated that the activated deletion mutants stimulated the myelin basic protein kinase activity of the MAP kinase substrate, confirming phosphorylation of the regulatory tyrosine and threonine residues (data not shown). These data demonstrate that expression of the mutationally activated MEK1 and MEK2 proteins results in morphological transformation of Rat1 cells and that transforming activity requires both constitutively elevated MEK activity and integrity of the PR sequence.

Like the wild-type protein, MEK1 S218/222D is capable of associating with c-Raf-1 in coimmunoprecipitation assays (data not shown) and pull-down experiments utilizing GST-MEK1 S218/222D (Fig. 2). Not surprisingly, the corresponding deletion mutant, MEK1 S218/222D Δ270-307, fails to associate

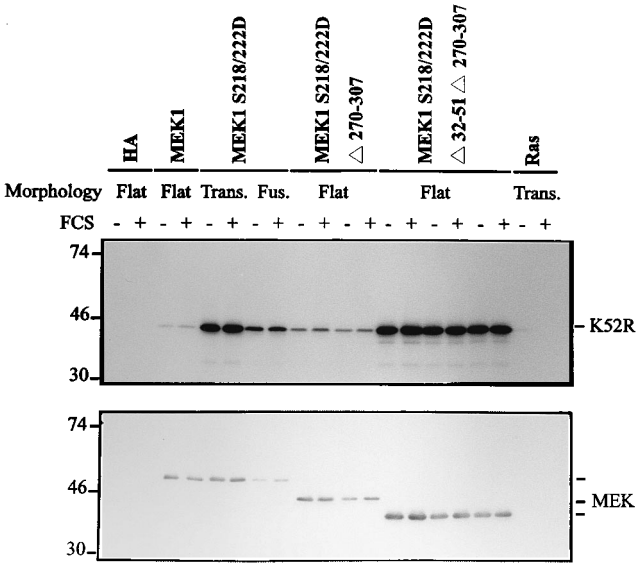

FIG. 8. Elevated MEK1 activity is not sufficient for transformation. Rat1 clones (see text) of the indicated morphology (Trans., florid transformation; Fus., fusiform transformation) were serum deprived and left untreated (−) or stimulated (+) with fetal calf serum (FCS) before lysis and immunoprecipitation with antisera specific for the HA tag. Immunoprecipitates were halved and either assayed for MEK activity (upper panel) as described in the legend to Fig. 3 or blotted for epitope-tagged MEK (lower panel). K52R denotes the position of phosphorylated MAP kinase substrate, and the positions of epitope-tagged MEK proteins are indicated right. Positions of size standards are indicated in kilodaltons at the left.

with c-Raf-1 in these assays (Fig. 2 and data not shown). The potential significance of these observations will be discussed below.

DISCUSSION

Numerous laboratories have participated in the identification of components of the MAP kinase pathway (reviewed in reference 46 and references therein). It is increasingly apparent that activation of MAP kinase is not the result of a simple linear sequence of enzyme reactions but rather involves regulation of protein-protein interactions and protein localization at multiple points, from recruitment of signalling molecules onto growth factor receptors at the cell periphery to translocation of MAP kinase to the nucleus. There is much circumstantial evidence to suggest that these protein-protein interactions confer specificity in signalling through structurally similar but functionally distinct kinase cascades.

The PR sequence is necessary for activation of MEK1. Recent data suggest that the activation of MEK1 proceeds through the formation of a ternary complex with Ras-GTP and Raf (30, 34, 42, 65). In this report, we show that disruption of the Raf-MEK1 complex by deletion of a PR sequence in MEK1 greatly inhibits basal and ligand-induced activation of MEK1. The same mutation also inhibits MEK1 activation in vitro by B-Raf, providing strong evidence that the PR sequence between subdomains IX and X is required for recognition and activation of MEK1 by Raf family enzymes. This might in part explain why a synthetic peptide based on the activating phosphorylation sites within MEK1 is a very poor substrate for c-Raf-1 (14): sequences distant to the phosphorylation sites are required for recognition of the substrate (76). However, MEK1 that lacks the PR sequence can be activated in vitro by a novel MEK activator partially purified from fibroblast cytosol (58). This activity may account for the residual MEK activity seen in antiepitope immunoprecipitates prepared from cells expressing the MEK1 Δ270-307 deletion mutant. It will be of interest to determine if activation of the deletion mutant by the unidentified cytosolic activator is dependent on Ras and/or Raf.

Since the PR sequence present in MEK1 appears important for efficient activation of the enzyme, one might speculate that this underlies the specificity of MEK versus SEK activation in vivo (18, 48): SEK1/MKK4, an activator of the JNK/SAP kinases, lacks this sequence and is poorly activated by oncogenic Raf in vivo (48, 75). This would provide a mechanism whereby cells could prevent activation of the JNK/SAP kinases by growth and differentiation factors. Importantly, since activation of the JNK/SAP kinases can occur in response to at least some agonists that stimulate apoptosis (18, 48), this mechanism might protect against inappropriate cell death. Insertion of the MEK PR sequence into SEK1/MKK4 might yield important information regarding the specificity of activation of MAP kinases versus JNK/SAP kinases.

Threonine 292 is necessary for ternary complex formation with Ras-GTP but dispensable for association with Raf. We previously demonstrated that MEK1 is able to form a ternary complex with Ras-GTP and Raf (34). Under these conditions, MEK1 T292A, which is mutant at a site of phosphorylation within the PR sequence, and MEK2, which lacks a phosphorylatable residue at the corresponding position, are not found in ternary complexes (34). Since Raf can associate directly with Ras-GTP (21, 50, 70–72, 77), these data suggested that threonine 292 might be necessary for association between MEK1 and Raf and that this could explain the failure of MEK2 to form a ternary complex with Ras. However, this appears not to be true, since both MEK2 and MEK1 T292A are able to interact with c-Raf-1. Experiments performed with the yeast two-hybrid system are supportive of this observation: both MEK2 and MEK1 T286/292A are able to associate with c-Raf-1 (data not shown). Hence, in our experiments, association between Raf-MEK and GTP-Ras apparently is regulated by additional factors that control formation of higher-order structures. Since c-Raf-1 is itself sufficient to bind GTP-Ras in vitro, we speculate that association of an unknown factor with Raf-MEK2 and Raf-MEK1 T292A is responsible for the inability of these proteins to interact with GTP-Ras. If this supposition is correct, phosphorylation of threonine 292 within the PR sequence of MEK1 might inhibit binding of this unknown factor to the Raf-MEK heterodimer, thereby allowing formation of the GTP-Ras-Raf-MEK1 ternary complex.

Threonine 292 regulates the kinetics of inactivation of MEK following cell stimulation. In addition to its role in activation of MEK1, elements within the PR sequence function in inactivation of MEK catalytic activity. Specifically, MEK2 and MEK1 T292A inactivate more rapidly than MEK1. A positive role for threonine 292 in maintenance of MEK1 activity following agonist stimulation is not supported by findings of Brunet et al., who report that phosphorylation of this site, perhaps by activated MAP kinase, correlates with feedback inhibition of MEK1 activity (5). In apparent contradiction to these results, we find that threonine 292 phosphorylation is constitutive: feedback inhibition of MEK1 activity by MAP kinase-catalyzed phosphorylation of this site is therefore unlikely in the cells used in these studies. Mansour et al. (45) and Saito et al. (61) arrived at the same conclusion after examination of MAP kinase phosphorylation of MEK1 in vitro. Consistent with a positive role for threonine 292 phosphorylation in maintenance of MEK1 activity, we previously reported that integrity of this site is necessary for association with immobilized GTP-Ras; neither MEK1 T292A nor MEK2 was found in these complexes (34). One attractive model is that phosphorylation of threonine 292 allows prolonged or repeated association with GTP-Ras, thus extending MEK1 activity relative to MEK1 T292A and MEK2. Indeed, since Raf-MEK2 does not form a complex with Ras-GTP (34) (see above), one might speculate that the Raf-MEK2 complex is activated by a distinct mechanism independent of Ras-GTP. Consistent with this suggestion, we find that MEK1 is preferentially activated in v-*ras*-transformed cells (34). A Ras-independent pathway for activation of MAP kinase has been suggested by Burgering et al. (6).

Recent work has prompted speculation that the timing and/or duration of MAP kinase activity may in part determine the biological response to agonist stimulation (35, 46). Thus, in PC12 cells, the mitogen EGF stimulates transient MAP kinase activity, whereas treatment with nerve growth factor (NGF) induces sustained MAP kinase activity and differentiation (28, 52, 67). Similarly, microinjection of a constitutively active MEK1 mutant in PC12 cells also results in differentiation (10). Our data suggest two mechanisms for these observations. One possibility is that the different agonists utilize distinct MEKs, with EGF using MEK2 and NGF using MEK1. Alternatively, both agonists might utilize MEK1, in which case prolonged MEK1 and MAP kinase activity might result from an NGF-induced phosphorylation of threonine 292. This model would predict that threonine 292 phosphorylation will not occur in response to EGF. Phosphorylation of this residue has been observed in MEK1 isolated from NGF-stimulated PC12 cells (61).

The PR sequence of MEK1 has an effector function. In addition to its regulatory role in activation and inactivation of MEK1, the PR sequence is essential for morphological trans-

formation by mutationally activated MEK1. Thus, although Rat1 cells expressing mutationally activated variants of MEK1 were morphologically transformed as described previously (10, 44), all clones expressing mutationally activated MEK1 but lacking the PR sequence were morphologically normal. Quantitative differences in MEK activity are not responsible for the different biological phenotypes, since the nontransforming MEK1 S218/222D Δ32-51 ΔPR sequence mutant had activity similar to that of MEK1 S218/222D, which was transforming.

One possible explanation for these results is that association of Raf with MEK1 allows appropriate localization of MEK1. It is plausible that activation of MAP kinase at specific sites within the cell is necessary for morphological transformation. There is some evidence that functional Ras is required for morphological transformation of NIH 3T3 cells by mutationally activated MEK1 (cited in reference 10), consistent with the possibility that Ras-mediated translocation of the Raf-MEK1 complex is important for signalling by mutationally activated MEK1. However, it is equally possible that paracrine or autocrine loops requiring functional Ras are necessary for morphological transformation by mutationally activated MEK1.

A second possibility is that complex formation modulates the efficiency of MAP kinase activation by MEK1. Disruption of the oligomeric complex by deletion of the PR sequence would thus attenuate signalling by mutationally activated MEK1 in vivo. Since deletion of the PR sequence inhibits association with Raf, Raf is a candidate molecule for this stimulatory function. However, other molecules that associate with MEK could serve the same function.

Finally, since transformation by MEK1 requires both constitutively elevated enzyme activity and a functional PR sequence, our data might indicate a dichotomy in signalling downstream of MEK1. Hence, it is possible that the PR sequence mediates interaction with an unidentified binding partner and/or substrate required for transformation by mutationally activated MEK1. In this regard, it is interesting that the MEK1 PR sequence contains a number of elements similar to consensus SH3 domain-binding motifs (9) and that one of these elements is flanked by phosphorylation sites specific to MEK1. However, none of these sequences has identity with known SH3 domain-binding sites, and it remains to be determined if these motifs serve as binding sites for cellular proteins.

In summary, our data indicate a critical role for the PR sequence of the MEKs in directing specific protein-protein interactions important for activation, desensitization, and downstream functioning. Identification of the molecular players in these roles is the subject of current work.

ACKNOWLEDGMENTS

A.D.C. and H.-J.S. contributed equally to this work.

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